ATTORNEY DOCKET NO. 02108.0001U2
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
Leonard et al.	)	
Application No. 09/708,352	)	
Confirmation No. 1597	)	Examiner: Ford, V.
Filed: November 8, 2000	)	Art Unit: 1645
For: **"Vaccines for Mycoplasma Bovis and Method of Use"**	)	

DECLARATION UNDER 37 C.F.R. § 1.132

NEEDLE & ROSENBERG, P.C.
The Candler Building
127 Peachtree Street, N.E.
Atlanta, Georgia 30303-1811

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Marvin F. Field, hereby declare that:

1. I am a retired research scientist, university professor and vaccine industry manager currently residing in Leawood, Kansas, and hold a Ph.D. in Microbiology from the University of Minnesota, a M.S. in Microbiology and in Biochemistry from Michigan State University, and a BA in Chemistry from Central Michigan College. I have over 45 years experience in the field of developing therapeutics with an emphasis on the study of pathogens and vaccines to protect against pathogens. This includes specific experience in vaccine development and testing. A partial curriculum vitae is attached to this declaration as an exhibit.

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2. I have reviewed the specification of the above-identified application.

3. I have reviewed the Office Action mailed June 18, 2002, in connection with the above-identified application and the following references cited in that Office Action:

a. "Protection against respiratory disease in calves induced by vaccines containing respiratory syncytial virus, parainfluenza type 3 virus, *Mycoplasma bovis* and *M dispar*," by Howard, Stott, Thomas, Gourlay and Taylor, cited as disclosing a vaccine containing the killed antigens of *M. bovis* and other pathogens; and

b. "Genomic, protein and antigenic variability of *mycoplasma bovis*," by Poumarat, Solsona, and Boldini, cited as disclosing the existence of marked intraspecies genomic heterogeneity among isolates of *Mycoplasma bovis* collected from different geographic origins and that antigenic variability must be taken into account in developing diagnostic and vaccination strategies.

4. I understand that claims 1-4 and 21 have been rejected under 35 U.S.C. § 102 as unpatentable over Howard et al. Specifically, I understand that the rejection is based in part on the contention that the composition of claims 1-4 and 21 has not been shown to not possess the same material structural and functional characteristics of the vaccine described in Howard et al. I present in this declaration evidence indicating that new claims 27 and 28 having specified amounts of *M. bovis* protein in a given volume do differ significantly from the vaccine described in Howard et al. Specifically, a sample of vaccine made according to the present invention containing 1.84×10^9 cell equivalents was determined by use of the Biorad reagent to contain 6

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micrograms of protein. From this, it is determined that the vaccine of Howard et al. that contains 500 micrograms of *M. bovis* protein as determined by the use of the Biorad reagent (see Howard et al., page 373, 1st column, 9th - 10th lines in section titled "Vaccines") must contain 1.5×10^{11} cell equivalents per vaccine dose. This is significantly, i.e., approximately 1500-fold, greater than the number of cell equivalents present in the vaccine dose described in the present application. Further, Howard et al. describes that the dose of vaccine be of a volume of approximately 5 mL. The presently claimed vaccine, as recited in claims 27 and 28, can be of a volume of 2 to 5 mL. Accordingly, the concentration of the presently claimed vaccine of claims 27 and 28 must necessarily differ from that of Howard et al. in that the composition is significantly, i.e., 600-1500 fold, less concentrated in regard to *M. bovis* cell equivalents (or *M. bovis* proteins).

5. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 18, 2002

Marvin F. Field
Marvin F. Field, Ph.D.

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Marvin F. Field
10316 Mohawk Road
Leawood, Kansas 66206
Telephone 913-648-5686

Professional Experience

1995-Present	Retired
1979-1995	Manager, Biologics Research and Development Sanofi Animal Health, Lenexa, Kansas Developed and licensed 25 new vaccines for swine and cattle. Winner of the Elf Aquitane Award for IBR Plus, a product for the protection of cattle against respiratory infections
1975-1979	Director, Vaccine Development Douglas Industries, Lenexa, Kansas Developed a new rabies vaccine for dogs and cats Licensed two other vaccines for cats
1970-1975	Manager, Biologics Research and Development Bayvet Company (Bayer), formerly known as Cutter Haver Lockhart Laboratories, Shawnee, Kansas Developed and licensed new vaccines for horses and cattle Researched new adjuvants for vaccines
1962-1970	Associate Professor, University of Missouri Kansas City and the University of Kansas Medical Center, Kansas City, Kansas Instructor of Microbiology to medical and dental students Initiated and performed research in electron microscopy at both institutions
1961-1962	Senior Research Scientist, Jensen Salsbury Laboratories, Kansas City, Kansas Researched hog cholera vaccines and canine distemper

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1957-1961	Manager, Biologics Quality Control, Chas. Pfizer and Company, Terre Haute, Indiana Developed testing methodology and assisted in licensing vaccines for Salk and Sabin Poliomyelitis, DPT and Measles
1951-1953	Senior Scientist, Chas. Pfizer and Company, Brooklyn, New York Researched various antibiotics

Educational History

1953-1957	Ph.D. in Microbiology, University of Minnesota
1948-1950	M.S. in Microbiology and in Biochemistry, Michigan State University
1944-1948	B.A. in Chemistry, Central Michigan College, Mt. Pleasant, Michigan

Professional Affiliations

- American Society for Microbiology (ASM)
- American Chemical Society (ACS)
- American Association for Advancement of Science (AAAS)
- Conference of Research Workers in Animal Diseases (CRWAD)

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Publications and Presentations

Field, M., K. Shedrick, J. Cullor, and G. Anderson
"Safety, Immunogenicity and Protection of Cattle Against Heterologous Endotoxin Challenge Following Vaccination with an E. coli J-5 Bacterin-Toxoid."
Conference of Research Workers in Animal Disease 1993 (abstract/poster)

Field, M., R. Ament, D. Lamb, and J. Blades
"Suckling Mouse Brain Rabies Vaccine (SMBV): Duration of Immunity in Dogs."
VM/SAC Pet Practice January 1976: pages 37-40

Rodriguez, A., and M. Field
"Pathogenesis of T-virus in Japanese Quail." Federation Proceedings 1971 (abstract)

Garrison, R., J. Lane, and M. Field
"Ultrastructural Changes During the Yeastlike to Mycelial Phase Conversion of Blastomyces dermatidis and Histoplasma capsulatum." Journal of Bacteriology 1970: Vol. 101

Lane, J., R. Garrison, and M. Field
"Ultrastructural Changes on the Yeastlike to Mycelial Phases of Sporotrichum schenckii." Journal of Bacteriology 1969: Vol. 100

Rodriguez, A., and M. Field
"Quantitative Studies on Reticuloendotheliosis virus Strain T."
Bacteriological Proceedings 1969

Kaplan, A., J. Davies, and M. Field
"The Hemodynamic Bisection of the Liver." Surgery 1969: Vol. 66 page 357

Field, M.
"Studies on a Fibrinolytic Enzyme from Monkey Renal Cells."
Bacteriological Proceedings 1966 (abstract)

Field, M.
"A Fibrin Overlay Technique." Bacteriological Proceedings 1964

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Crawford, J., and M. Field
"Potency Values of Poliomyelitis Vaccines by an Antibody Combining Test."
Journal of Bacteriology 1960: Vol. 80 pages 111-118

Field, M., and H. Lichstein
"Growth Stimulating Effect of Autoclaved Glucose Media and its Relationship to the CO_2 Requirement of Propionic Acid Bacteria."
Journal of Bacteriology 1958: Vol. 76 pages 485-490

Field, M., and H. Lichstein
"Influence of Casein Hydrolysates and Amino Acids on Glucose Fermentation by Propionibacterium freudenreichii."
Journal of Bacteriology 1958: Vol. 76 pages 491-494

Field, M.
"Factors Affecting the Growth of Propionibacteria." Journal of Bacteriology 1957: Vol. 73 page 92 (abstract)

English, A., and M. Field
"Carbomycin IV: Tissue Distribution in the Rabbit." Antibiotics Annual 1953/1954: pages 522-525

English, A., and M. Field
"Magnamycin III: Preliminary Studies on Absorption and Excretion after Single Dosage." Antibiotics and Chemotherapy 1953: Vol. 3 pages 307-313

Field, M., and A. English
"Magnamycin I: In Vitro Studies." Antibiotics and Chemotherapy 1952: Vol. 2 pages 678-688

Field, M.
"Qualitative and Quantitative Studies of Vibrio fetus Antigen Produced in Various Media, with Emphasis on the Efficacy of a Fluid Medium."
Master Theses, Michigan State University (1950)

Huddleson, I., and M. Field
"A Satisfactory Medium for the Isolation, Cultivation and Maintenance of Viability of Vibrio fetus." Journal of Bacteriology 1948: Vol. 56 page 508

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#16 2 Moon 1/2/03

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Commissioner for Patents
Washington, D.C. 20231

NEEDLE & ROSENBERG, P.C.
The Candler Building
127 Peachtree Street, N.E.
Atlanta, Georgia 30303-1811

Sir:

I, Dr. Ping Wu, hereby declare that:

1. I am a Research Scientist at Biomune, in Lenexa, Kansas, and hold a Ph.D. in Pathology from Michigan State University, a M.S. in Veterinary Medicine from Huazhong Agricultural University, and a BS (DVM equivalent) in Veterinary Medicine from Fujian Agricultural University. I have over 15 years experience in the field of animal health, with an emphasis on the study of pathogens and vaccines to protect against pathogens. This includes specific experience in vaccine development and testing. A partial curriculum vitae is attached to this declaration as an exhibit.

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2.. I have reviewed the specification of the above-identified application.

3. I have reviewed the Office Action mailed June 18, 2002, in connection with the above-identified application and the following references cited in that Office Action:

a. "Protection against respiratory disease in calves induced by vaccine containing respiratory syncytial virus, parainfluenza type 3 virus, *Mycoplasma bovis* and *M dispar*," by Howard, Stott, Thomas, Gourlay, and Taylor, cited as disclosing a vaccine containing the killed antigens of *M. bovis* and other pathogens; and

b. "Genomic, protein and antigenic variability of *mycoplasma bovis*," by Poumarat, Solsona, and Boldini, cited as disclosing the existence of marked intraspecies genomic heterogeneity among isolates of *Mycoplasma bovis* collected from different geographic origins and that antigenic variability must be taken into account in developing diagnostic and vaccination strategies.

4. I understand that claims 5-12 and 21 have been rejected under 35 U.S.C. § 103 as unpatentable over Howard et al. in view of Poumarat et al. Specifically, I understand that the rejection is based in part on the contention that Poumarat et al. teaches the existence of different genotypes of *M. bovis* and that the antigenic variability represented by differences in *M. bovis* genotypes must be taken into account when preparing vaccines otherwise in accordance with Howard et al. I present in this declaration evidence indicating that the isolates of Poumarat et al., what are referred to by the Examiner as "biotypes," do not represent the biotypes of the present invention as claimed in claims 5-12, and 24. Specifically, as shown in the attached exhibit,

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labeled "*Pst*I Digestion Patterns," the pattern of cleavage exhibited by biotypes A, B and C of the present invention, do not correspond to those of the isolates tested by Poumarat et al. (a copy of page 309 from Poumarat et al. is also attached for comparison). Accordingly, it can be established that the "biotypes" identified by Poumarat et al. are not biotypes A, B, or C of the present invention.

Genomic DNA from four *M. bovis* isolates, identified as 108-97 (biotype A), 275-99 (biotype C), 280-99 (biotype B) and 282-99 (biotype A) was isolated using QIAGEN's DNeasy Tissue Kit. 10 micrograms of each of the genomic DNAs were digested with *Bam*HI, *Pst*I, and *Sma*I overnight at 37°C. The resulting DNA fragments were separated by horizontal gel electrophoresis using a 1% agarose gel in TAE buffer at 100 volts for 3 hours. These conditions are conventionally used in REA and are functionally equivalent to those used by Razin et al. which is cited in Poumarat et al.'s paper (Razin et al. labeled Attachment B, see page 1937).

The resulting gels provide REA patterns (such as that shown in the exhibit labeled "*Pst*I Digestion Patterns" which depicts REA patterns generated from *Bam*HI, *Pst*I and *Sma*I digestion of four *M. bovis* isolates). Lanes 1-4, isolate 280-99 (biotype B); Lanes 5-8, isolate 282-99 (biotype A); Lanes 9-12, isolate 275-99 (biotype C), Lanes 13-16, isolate 108-97 (biotype A); and Lane 17, a molecular marker (100 bp ladder from BioRad); are described herein. Lanes 1, 5, 9 and 13 are controls containing undigested *M. bovis* DNA. Lanes 2, 6, 10 and 14 contain *M. bovis* DNA digested with *Bam*HI. Lanes 3, 7, 11 and 15 contain *M. bovis* DNA digested with *Pst*I. Lanes 4, 8, 12 and 16 contain *M. bovis* DNA digested with *Sma*I.

Analysis of the patterns of fragments obtained and comparison of these to the results published in Poumarat et al., demonstrate the biotypes A, B and C differ from those of Poumarat et al. While it was not possible to relate the patterns of these biotypes' *Bam*HI and *Sma*I patterns

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to those of Poumarat et al., because the there is no data relating to *Bam*HI and *Sma*I patterns in the paper, the *Pst*I REA patterns obtained for biotypes A, B, and C are different from the 5 *Pst*I patterns shown in Figure 1 of Poumarat et al.'s paper. Thus, the presently described biotypes A, B and C were not disclosed in Poumarat et al.

5. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12/18/02

Ping Wu
Ping Wu, Ph.D.

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Dr. Ping Wu
14275 W. 147th Terrace • Olath • KS 66062 • (913) 939-0435

SUMMARY

More than 4 years of biologic industrial experience (R&D department) as research scientist with achieving three USDA licenses {1 conventional MDV vaccine (team member) and 2 genetically modified HVT vectored vaccines (major investigator)}. 6 years of training and experience in molecular biology, molecular virology, and avian pathology. 6 years of experience on virology, immunology, cell biology, and diagnostic technology. Research interests: apply my knowledge and experience to advanced research and development of biological products.

EMPLOYMENT

4/2002 to Present — **RESEARCH SCIENTIST**
Biomune Company, Lenexa, KS

10/98 - 4/02 — **RESEARCH SCIENTIST**
Tri Bio Labs. Inc., Hoechst Roussel Vet Corporation
State College, PA

9/94 - 9/98 — **GRADUATE RESEARCH ASSISTANT**
Michigan State University, East Lansing, MI

7/92 -9/94 — **VISITING SCIENTIST**
USDA-ARS Avian Disease and Oncology Laboratory
East Lansing, MI

12/85 – 7/92 — **RESEARCH ASSISTANT**
Fujian Academy of Agricultural Science. Fuzhou, Fujian, China

2/82 – 9/83 — CLINICAL VETERINARIAN
Wuyishan City Vetinarary clinical station, Wuyishan City, Fijian, China

EDUCATION

Michigan State University East Lansing, MI
Ph.D., Pathology, 1998
Major Molecular Virology and Avian Pathology

Huazhong Agricultural University, Wuhan, Hubei, China
M.S., Veterinary medicine, 1985
Major Immunopathology

Fujian Agricultural University, Fuzhou, Fujian, China
B.S. (DVM equivalent), 1982
Major Veterinary Medicine

EXPERIENCE
10/98 – present

RESEARCH SCIENTIST
Focus of research: Research and development of genetically modified and conventional poultry virus vaccines

- Following USDA guideline, responsible to design and conduct bioanalytical assays for assessing vaccine candidate viruses, including genetically modified viruses and conventional attenuated viruses.
- Responsible to collect, analyze, and validate experimental results.
- Responsible to prepare research reports for submitting to USDA.
- Interaction with scientists at central for Veterinary biologics and national veterinary services laboratory of USDA for scientific issues.
- Supervise 2 technicians and 2 animal caretakers at R & D Department.

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7/92 - 9/98

GRADUATE RESEARCH ASSISTANT AND VISITING SCIENTIST
Focus of research: Genomic organization and gene functions of Marek's virus, an alpha-herpesvirus.

Dissertation research focuses on functional analysis of glycoprotein H and L complex of Marek's disease virus.

DNA and RNA related experience
- Genomic DNA and RNA preparation.
- PCR and RT-PCR.
- Genomic and cDNA cloning, plasmid DNA preparation.
- Sequencing and data assembly and analysis.
- Southern and Northern hybridization
- Computer skill on molecular biology. MacVector, DNAStar, Gene construction kit, Oligo, GCG package, Blast in NCBI
- Database search and gene minning.

Protein expression and purification
- Expression and purification of *TrpE* and *GST* fusion proteins in *E-coli* expression system
- Transient expression and analysis of foreign protein in cell culture by T7 RNA polymerase.
- Expression and analysis of foreign protein with virus vectors (fowlpox virus and baculovirus)
- Immunoprecipitation and Western blot assay for protein analysis

12/85 - 6/92.

RESEARCH ASSISTANT
Focus of research: Development of monoclonal antibodies against animal viruses, development of assays for avian disease diagnosis

Cell culture
- Primary cell culture (CEF, CK, DEF, RK)
- Cell lines. BHK21, PK15, OU2, DF1, Vero, COS1 and COS7, NS-1 and SP2/O, hybridoma cell lines (monoclonal antibody secreting cell lines)
- Propagation and purification of animal viruses (PRV, MDV, IBDV, ILTV, NDV, IBV)

Serological methods
- ELISA
- Indirect immunofluorescence assay (IFA)
- Immunohistochemistry assay.

MEMBERSHIP

- American Veterinary Medical Association (AVMA)
- American Association of Avian Pathologist (AAAP)
- Animal Health Institute of USDA (Vet. Biologicals section)

HONORS

1998

Award from U.S. Department of Agriculture for the outstanding contribution to the understanding of genomic structure of Marek's disease virus.

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PUBLICATIONS

Lee L. F., **Wu P.**, Sui D., Ren D., and Kung H-J. The complete UL sequence of serotype I Marek's disease virus. GenBank, Accession number AF147806, 1999. (Will be released in December 31, 1999).

Wu P., Lee L. F., and Reed M. W. Glycoprotein H and L of Marek's Disease Virus Form A Hetero-oligomer, and gL is Required For gH Cell Surface Expression. Submitted to Virology, 1999.

Wu P., Lee L. F., and Reed M. W. Identification and Characterization of Glycoprotein H of MDV GA Strain. Acta Virologica. 43:152-158, 1999

Cui Z., Qin A., Lee F. L., **Wu P.** and Kung H. –J. Construction and characterization of a H19 epitope point mutation of MDV CVI988/respens strain. Acta Virologica. 43:169-173, 1999

Reddy S. M., Sui D., **Wu P.** and Lee F. L. Identification and structural analysis of a MDV gene encoding a protein kinase. Acta Virologica. 43:174-180, 1999

Wu P., Lee L. F., and Reed M. W. Serological characteristics of a membrane glycoprotein gp82 of Marek's Disease virus. Avian Disease. 41:824-831. 1997.

Sui D., **Wu P.**, Kung H-J., and Lee F. L. Identification and characterization of a Marek's disease virus Gene encoding DNA polymerase. Virus Research. 36:269-78. 1995

Wu P., Li Y., Ling T., Zhuang X., Huang N., and Cheng Y. Characterization and application of monoclonal antibodies to pseudorabies. Chinese J. of Virology. NO. 2, 1992.

Wu P., Ling T., Zhuang X., Li Y., Huang N., and Cheng Y. Comparison among four methods to detect pseudorabies virus antigen. Chinese J. of Veterinary Medicine. No. 1, 1992.

Wu P., Zhuang X., Ling T., Li Y., Huang N., and Cheng Y. Application of reserve passive heamaglutination and its inhibition in detecting pseudorabies. Chinese J. of Vet. Sci. and Technol. No. 11, 1991.

Zhuang X., **Wu P.**, Ling T., Li Y., Huang N., and Cheng Y. Microball double antibody sandwich ELISA for the detection of Newcastle Disease virus antigen in chickens. J. Fujian Academy of Agri. Science. 6 (1) : 27, 1991

Wu P., Li Y., Zhuang X., Huang N., Ling T., and Cheng Y. A direct enzyme-linked immunosorbent assay for the detection of pseudorabies virus antigen in mice. J. Fujian Academy of Agri. Science. 5 (1) : 3, 1990.

Wei Z. M., **Wu P.**, Dai C., Li Y., and Cheng Y. Studies of attenuated pseudorabies virus vaccine – its effect on immunizing cattle and goats. J. Fujian Academy of Agri. Science. 5 (1) : 93, 1990.

Wu P., Wei Z. M., Dai C., Li Y., and Cheng Y. Studies of attenuated pseudorabies virus vaccine – cellular immune response in the inoculated swine. Chinese J. of Vet. Sci. And Technol. No. 11, 1990.

Cheng Y., **Wu P.**, Li Y., Zhuang X., Huang N., and Ling T. Preparation of pseudorabies virus monoclonal antibody-peroxidase conjugate and its application to detection of PRV antigen in mice. Agricultural Biotechnology in China. p172 - 177, 1989.

Cheng Y., Li Y., Ling T., Hang N., and **Wu P.** Characterization and application of monoclonal antibodies to Newcastle disease virus. Chinese J. of Virology. 3 (4) : 332, 1988.

PROCEEDINGS

Wu P., Sui D., and Lee L. F. Nucleotide sequence analysis of a 9-kb region of Marek's disease virus genome exhibits a collinear gene arrangement with the UL29 to UL36 genes of herpes simplex virus. Current Research on Marek's Disease. Silva R. F., Cheng H. H., Coussens P. M., Lee L. F., and Velicer L. F., Edited, Proceeding of the 5th international symposium on Marek's disease, p219-224. East Lansing, Michigan, USA. Sept. 1996.

Lee L. F., **Wu P.**, and Sui D. Identification and transcriptional analysis of a Marek's disease virus gene encoding membrane glycoprotein gp82. Current Research on Marek's Disease. Silva R. F., Cheng H. H., Coussens P. M., Lee L. F., and Velicer L. F., Edited, Proceeding f the 5th international symposium on Marek's disease, p219-224. East Lansing, Michigan, USA. Sept. 1996.

Mao Y., **Wu P.**, Hung H-J., and Lee L. F. DNA sequ nce analysis of MDV genomic fragments BamHI-P2, -I1, -J and EcoRI-E reveals a large open reading frame homologous to a herpes simplex virus gene

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encoding a DNA binding tegument protein. Current Research on Marek's Disease Silva R. F., Cheng H. H., Coussens P. M., Lee L. F., and Velicer L. F., Edited, Proceeding of the 5th international symposium on Marek's disease, p219-224. East Lansing, Michigan, USA. Sept. 1996.

PRESENTATIONS

Lee F. L., **Wu P.**, Hunt H., and Fadly A. Biological properties of subgroup-J avian leukosis virus envelope glycoprotein. American Veterinary Medical Association, 136th annual convention. New Orlance. July 1999

Qin A., Lee F. L., and **Wu P.** Development and characterization of monoclonal antibodies to avian leukosis virus subgroup-J. American Veterinary Medical Association, 136th annual convention. New Orlance. July 1999 **(AAAP 1999 annual convention best poster award)**

Wu P., Lee F. L., and Reed M. Willie. Expression of MDV gH and gL in recombinant baculovirus infected cells. American Veterinary Medical Association, 135th annual convention. Baltimore. July 1998

Wu P., Lee L. F., and Reed M. W. Characterization of a MDV gene homologous to glycoprotein H of herpes simplex virus. American Veterinary Medical Association, 134th annual convention. Reno. July 1997.

Wu P., Lee L. F., and Reed M. W. Identification and characterization of a Marek's disease virus gene homologous to UL21 of herpes simplex virus. American Veterinary Medical Association, 133rd annual convention. Louisville. July 1996.

Wu P., Lee L. F., and Reed M. W. Characterization of a monoclonal antibody to Marek's disease virus membrane protein. American Veterinary Medical Association, 132nd annual convention. Pittsburgh. July 1995.

Lee L. F., and **Wu P.** Identification of a small subunit of ribonucleotide reductase gene of Marek's disease virus. American Veterinary Medical Association, 132nd annual convention. Pittsburgh. July 1995.

Wu P., Sui D., and Lee L. F. Identification of a Marek's disease virus gene homologous to UL32 of herpes simplex virus. American Veterinary Medical Association, 131st annual convention. San Francisco. July 1994.

Sui D., **Wu P.**, and Lee L. F. Identification and characterization of a Marek's disease virus gene essential for virus replication. American Veterinary Medical Association, 131st annual convention. San Francisco. July 1994.

Sui D., **Wu P.**, and Lee L. F. Marek's disease virus gene encoding glycoprotein H. American Veterinary Medical Association, 130th annual convention. Minneapolis. July 1993.

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Attorney Docket No. 02108.0001U2
Attachment A – Page 1 of 6

December 15,

The Production of Polyclonal Antibodies in Laboratory Animals

The Report and Recommendations of ECVAM Workshop 35[1,2]

Reprinted with minor amendments from *ATLA* **27**, 79-102.

Appendix 2

Overview of Adjuvants

An overview of adjuvant categories used for routine polyclonal antibody (pAb) production is given in Table I.

Table I: Overview of Categories of Adjuvants that May Be Used for Routine Polyclonal Antibody Production

Category	Examples (references)
Immunostimulatory oil emulsions (for example, water-in-oil, oil-in-water, water-in-oil-in-water	Freund's incomplete adjuvant, Montainde®, Specol (18)
Mineral salts	$Al(OH)_3$, $AlPO_4$
Microbial (like) products	LPS, MDP, MPL, TDM (10)
Saponins	Quil A (19)
Synthetic products	DDA (13), ISCOMs, NBP (12)

*

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Adjuvant formulations	Oil emulsion + NBP (TiterMax™), Oil emulsion + bacterial products (Freund's complete adjuvant; RIBI™, Gerbu (11)

LPS = lipopolysaccharide; MDP = muramyl dipeptide; MPL = monophosphoryl lipid A ; TDM = trehalose dimycolate; DDA = dimethyldioctadecylammonium bromide; NBP = non-ionic block polymer; ISCOMs = immune stimulating complexes.

Immunostimulatory Oil Emulsions

Water-in-oil emulsions, which include Freund-type adjuvants, are the adjuvants most commonly used to produce pAbs in laboratory animals. Although most investigators and commercial vendors still refer to the Freund-type adjuvants in use today as Freund's complete adjuvant (FCA, i.e. it contains mycobacteria) or Freund's incomplete adjuvant (FIA, i.e. it does not contain mycobacteria), it would be desirable to replace these terms to reflect the differences between the components in the original formulation and those in the modern formulation, as well as the differences in reactogenicity of the different formulations. Few laboratories would be in a position to make the original FCA, because it was formulated with heat-killed *Mycobacterium tuberculosis* of high virulence, a mineral oil of low quality manufactured before 1969, and a surfactant, predominantly mannide mono-oleate, of variable purity and quality. Due to a change in the oil refining procedure in the early 1970s, the mineral oil component of the original FCA is no longer available (1). It has been replaced by a higher quality oil with less-irritant properties. The mannide mono-oleate currently in use is also of higher quality. Today, only a few adjuvant immunologists retain the use of the old FCA, which is manufactured by the StatensSerum Institute (Copenhagen, Denmark) as a "gold standard" for comparison against a new adjuvant. The workshop participants agreed that this formulation is unsuitable as an adjuvant for use in routine pAb production, due to severe side-effects. The product that is quoted as FCA in the more recent literature can be obtained from, for example, Difco Laboratories, Sigma, ICN Biomedicals, and Pierce, and consists of a refined oil and a high quality mannide mono-oleate preparation, with heat-killed *M. butyricum* or *M. tuberculosis* H37Ra, an avirulent human strain. This FCA has less-irritant and less-inflammatory properties than the original FCA, but still induces considerable side-effects in animals (2, 3).

Currently, there are immunostimulatory oil emulsions that are acceptable or even superior to the original FIA with regard to enhancing antibody responses. Moreover, the purlfied components in these formulations produce fewer and less-severe adverse reactions after injection, for example, the Montanide® ISA (Incomplete Seppic Adjuvant) series (Seppic, Paris, France) and NUFCA Guildhay oil (Guildhay, Guildford, Surrey, UK).

Montanide ISA 740 adjuvant is composed of highly purlfied mannitol octadecenoic esters (Montanide ISA 80) as surfactant, in a mixture of a metabolisable oil and a refined non-metabolisable light mineral oil classified pharmacologically as an excipient. This mixture can form a stable emulsion (especially under nitrogen storage), in the weight ratio 70:30 Montanide ISA 740:aqueous phase antigen. When properly formulated, the emulsion will remain in a single phase for at least 2 years. This adjuvant emulsion is easy to inject and is well-tolerated by the recipient animal. When injected subcutaneously into mice or guinea-pigs in accordance with the European Pharmacopoeia, there are no serious adverse effects. The Montanide ISA series has been accepted for use in all food-producing

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species (4), as a pharmacologically active substance generally regarded as safe.

A non-ulcerative oil (NUFCA Guildhay oil) that can be administered by the intramuscular, subcutaneous or intradermal routes at multiple sites has been introduced by Guildhay. The intramuscular site creates a focus of stimulus with fewer adverse reactions than the classical FIA on the market today. However, as NUFCA Guildhay oil has only recently been introduced, limited information on its use is currently available.

Mineral Salts

Aluminum adjuvants in the form of aluminum hydroxide or aluminum phosphate hydrated gels can be injected subcutaneously or intramuscularly for priming an immune response in the recipient. These adjuvants are generally regarded as safe and they have been used for human vaccination for more than 50 years (5). Priming immunizations with aluminum adjuvants can be followed by boosters witt or without adjuvant (6, 7). The biological function of these adjuvants is related to their ability to adsorb protein antigens, thereby ensuring that soluble proteins will be taken up as particulate antigens by antigen-presenting cells (8). Due to this adsorption/function relationship, it is strongly recommended that investigators ascertain that adsorption of the antigen to the gel has been successfully accomplished prior to its injection (9).

Microbial (like) Products

Micro-organisms such as *M. butyricum* and microbial products can exhibit strong adjuvant activity. The innate vertebrate immune system has evolved mechanisms for the recognition of, and response to, certain microbial products. Although the innate immune system itself is not highly efficient, some of its response components, once stimulated, help energise the specific antibody response. The microbial products involved (primarily cell wall components) usually induce considerable undesirable inflammatory side-effects, as well as an adjuvant effect. Investigators have identified active fractions or subunits of bacterial products, for example, trehalose dimycolate, and have in some cases mod)fied the bacterial products, for example, threonyl-muramyl dipeptide, or monophosphoryl lipid A, tc achieve a balance of immunostimulatory properties and diminished inflammatory properties (10, 11).

Saponins

Saponins are triperpene glycosides which are derived from the bark of the *Quillaja saponaria* tree and which have detergent and adjuvant properties. Saponin preparations intended for use as immunological adjuvants (for example, Quil A or QS-21) are purlfied to reduce the presence of components which cause adverse local reactions. Food-grade saponin preparations should not be used for immunization schemes. In general, saponins should not be injected intraperitoneally or intravenously, but only subcutaneously or intramuscularly, due to their haemolytic activity. *

Synthetic Pr ducts

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Synthetic adjuvants are a rather heterogeneous group of products, because their classification has no single chemical, physical, or functional basis. This group includes nonionic block polymers (NBP), dimethyldioctadecylammonium bromide (DDA), immune stimulating complexes (ISCOMs), and liposomes. NBPs can contain different hydrophobic and hydrophilic regions, which influence their surfactant and immunopotentiating properties (12). The adjuvant effect of a given NBP also depends on the antigen used in combination with it, and, as such, different NBPs may be needed for different antigens for optimal effects. DDA is not an optimal adjuvant for antibody responses (13), but is rather better for Tcellmediated cytotoxic responses. DDA has a lipophilic character, which might be responsible for its capacity to enhance T-cell responses. It is a representative of the quaternary amines (also classified as a cationic detergent). ISCOMs are small (40 nm diameter) cage-like structures prepared from Quil A, cholesterol, and phospholipids. The antigen to be inserted into ISCOMs must be amphipathic (14). ISCOMs can be recommended as an excellent first choice for viral vaccines, based on past successes. In part, this is because ISCOMs can deliver the antigen to the cytosolic compartment of antigenprocessing/antigen-presenting cells, and thus direct the immune response to a cytotoxic T-cell response, which is effective against many viruses. However, ISCOMs may also facilitate antibody responses. There is generally some resistance to the use of ISCOMs, because of the perceived, but misconceived, difficulty in their preparation; as an alternative, there are commercially available "honeycomb structures", to which the antigen can be added (15). Liposomes are unilamellar or multilamellar vesicles artificially constructed from natural products. The bilayer membranes mimic those of cells. The adjuvanticity of liposomes is influenced by charge, composition, and method of preparation. The antigen can be encapsulated in the water phase or the lipid phase, or it can be coupled to the surface (see reviews by Buiting *et al* [16] and Alving [17]).

Adjuvant Formulations

Combining different immunostimulatory agents can increase the potency of an adjuvant. Oil emulsions are frequently combined with other agents (for example NBP in TiterMax™, or bacterial products in FCA and RIBI™ adjuvants). Immunostimulatory agents such as muramyl dipeptide can be incorporated along with antigen into liposomes. In fact, many adjuvants have more than one immunostimulatory substance and more than one mechanism of action.

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DNA Cleavage Patterns as Indicators of Genotypic Heterogeneity among Strains of *Acholeplasma* and *Mycoplasma* Species

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Electrophoretic patterns of digestion products of *Acholeplasma* and *Mycoplasma* DNA by restriction endonucleases were compared. The patterns of *Acholeplasma axanthum* strains isolated from a variety of hosts and habitats differed markedly from each other, indicating considerable genotypic heterogeneity among strains included in this species. Heterogeneity was less marked among the *Acholeplasma oculi* strains tested, and was minimal among strains of the avian pathogen *Mycoplasma gallisepticum*. Strains of *Mycoplasma genitalium* isolated from the urethra of patients with non-gonococcal urethritis and from the urethra of an experimentally infected chimpanzee yielded identical cleavage patterns, indicating a high degree of genetic homogeneity of these strains. The data support the notion that mycoplasma species of strict host and tissue specificity exhibit marked genetic homogeneity. The advantages and deficiencies of the use of DNA cleavage patterns for classification purposes are discussed.

INTRODUCTION

The current concept of species in prokaryotes is ill-defined. Ideally, a prokaryotic species should consist of a cluster of strains of identical or nearly identical genetic composition. However, it is apparent that in the process of evolution, mutagenesis and selective pressures have resulted in the appearance of strains differing genotypically to varying degrees from the original strain, a factor which constitutes the major obstacle in bacterial classification. In practice, most prokaryotic species have been established on the basis of a few, readily-determined phenotypic characteristics, in the absence of supportive genetic data. In most cases this somewhat arbitrary speciation proved to be effective, serving the purpose of distinguishing bacteria of special medical, industrial and agricultural importance. Nevertheless, it is clear that in order to put bacterial classification on a more scientific basis, speciation should be determined according to genetic relatedness. Direct analysis of the base composition and nucleotide sequence of the bacterial chromosome may provide the necessary data. A variety of DNA-DNA hybridization techniques have been employed in assessment of genetic relatedness among prokaryotic strains, as these provide a measure of the degree of homology of nucleotide sequences between the chromosomes of the tested organisms. DNA base composition analysis and hybridization tests have indeed become useful tools in classification of prokaryotes, including mycoplasmas (Somerson *et al.*, 1967; Aulakh *et al.*, 1979, 1983; Stephens *et al.*, 1983).

We have recently promoted the use of cleavage patterns of mycoplasmal DNA digested by restriction endonucleases as 'finger-prints' indicating genetic relatedness among mycoplasma strains (Razin *et al.*, 1983; Chandler *et al.*, 1982). These enzymes cleave double-stranded DNA at specific recognition sites consisting of four or six nucleotides arranged in a specific order. The

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electrophoretic patterns of the resulting oligonucleotide fragments exhibit highly reproducible patterns specific for strains and species. By using this method nine serotypes of *Ureaplasma urealyticum* were shown to fall in two distinct clusters representing apparently two genotypes, whereas the DNA from five representative strains of *Mycoplasma pneumoniae* produced very similar cleavage patterns indicating the marked genetic homogeneity of this species (Chandler *et al.*, 1982; Razin *et al.*, 1983). Species specific cleavage patterns were also reported for the DNA of *Spiroplasma citri* strains cleaved by *Eco*RI (Bove & Saillard 1979; Bove *et al.*, 1982) and for *Mycoplasma hyorhinis* DNA digested by a variety of restriction endonucleases (Darai *et al.*, 1981, 1982).

Recent DNA-DNA hybridization tests (Aulakh *et al.*, 1983) revealed low nucleotide sequence homology (2 to 21%) among the eight established species of *Acholeplasma*, providing genetic support for their distinction. However, considerable variation in nucleic acid homology (ranging between 48 to 100%) was found among strains within each of the species *Acholeplasma laidlawii* and *Acholeplasma axanthum* (Stephens *et al.*, 1983). It seemed worthwhile to test whether or not this intraspecies genotypic heterogeneity can be observed by the restriction endonuclease method. Strains of *A. axanthum*, for which hybridization data were available, were selected for our study. Strains of *Acholeplasma oculi*, the avian pathogen *Mycoplasma gallisepticum* and the newly discovered human genital mycoplasma *Mycoplasma genitalium* (Tully *et al.*, 1981, 1983) were also included. The data presented here show that DNA cleavage patterns can be used as a convenient measure of intraspecies genotypic homogeneity or heterogeneity and support the notion that mycoplasma species of strict host and tissue specificity exhibit marked genetic homogeneity.

METHODS

Organisms and growth conditions. The designation of the strains tested and their origin are listed in Table 1. All the strains were cloned by the filtration procedure (Subcommittee on the Taxonomy of Mollicutes, 1979). The acholeplasmas and the *Mycoplasma gallisepticum* strains were grown in 200–400 ml quantities of 1% (w/v) bovine serum fraction broth medium (Tully & Razin, 1969) for 24–48 h at 37 °C, and harvested by centrifugation at 27000 *g* for 30 min at 4 °C. The cell pellets of the acholeplasmas were washed once in 0·25 M-NaCl containing 0·1 M-Na_2EDTA while those of *M. gallisepticum* were washed in 0·25 M-NaCl alone. The washed pellets were kept at −70 °C until used for DNA extraction. The *Mycoplasma genitalium* strains were grown in plastic tissue culture flasks (T-175) containing 200 ml SP4 medium (Tully *et al.*, 1977). The bottles were incubated horizontally at 37 °C for 88 h. The medium was decanted and the surface attached growth was washed twice with 10 ml 0·25 M-NaCl and then scraped off into another 10 ml of the salt solution. The organisms were collected by centrifugation at 18000 *g* for 10 min, and the cell pellets were used immediately for DNA extraction.

DNA extraction and purification. Portions of the washed cell pellets (containing about 20–50 mg cell protein) were resuspended in 0·1 ml TE buffer (50 mM-Tris, pH 8·0 and 10 mM-EDTA) and the organisms were lysed by adding 1 ml 1% (w/v) SDS in TE buffer. To minimize self-digestion of DNA by the endogenous DNAases of acholeplasmas (Razin *et al.*, 1964; Pollack *et al.*, 1965) the concentration of EDTA in the TE buffer was raised to 20 mM when acholeplasma pellets were lysed. The lysate was treated for 30 min at 37 °C with preheated (boiled for 2 min) RNAase A (Worthington Biochemical Corporation) at a final concentration of 50 μg ml^{-1}, and then for 60 min at 37 °C with preheated proteinase K (Boehringer) at 100 μg ml^{-1}. The SDS in the lysate was then precipitated by adding 100 μl 5 M-potassium acetate and incubated in ice for 30 min. The precipitate was sedimented by centrifugation at 15000 r.p.m. for 10 min in an Eppendorf microcentrifuge. The supernatant fluid was transferred to another microcentrifuge tube and mixed with an equal volume of redistilled phenol/chloroform (1:1, v/v) mixture. The aqueous phase was separated by centrifugation at 15000 r.p.m. for 15 min in the microcentrifuge and the DNA was precipitated by adding two volumes of cold ethanol. The DNA precipitate was collected by centrifugation for 5 min in the microcentrifuge. The resulting pellet was dried under vacuum and dissolved in 25–100 μl 10 mM-Tris, pH 7·5 containing 1 mM-Na_2EDTA. A sample (5 μl) of the DNA solution was diluted with 0·8 ml phosphate-buffered saline, and the absorbance was measured at 260 and 280 nm. The values of A_{260}/A_{280} were about 1·7 to 2·0, indicating the purity of the DNA preparations. The DNA content of the original solution was calculated according to the A_{260} value of the diluted solution, assuming that an A_{260} of 1·0 corresponds to 50 μg DNA ml^{-1} (Archer *et al.*, 1981).

DNA digestion. Restriction endonucleases were purchased from New England Biolabs (Beverley, Mass.). The reaction mixtures (total volume 20 μl) contained about 5 μg mycoplasmal DNA, 20–40 units of the tested endonuclease, 10 mM-Tris/HCl, pH 7·5, 10 mM-$MgSO_4$ and 50 mM-NaCl for digestion by *Bam*HI, *Pst*I and

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S, the lysate was then precipitated
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Table 1. *Designation and origin of strains tested*

Species and strain designation	Isolated from:	Isolated by:	Received from:
Acholeplasma oculi			
19L	Goat, eye	J. M. Al-Aubaidi	J. M. Al-Aubaidi
3557 TC	Tissue culture	R. A. DelGiudice	R. A. DelGiudice
Goat 5	Goat, eye	O. H. V. Stalheim	O. H. V. Stalheim
70-538 TC	Tissue culture	R. A. DelGiudice	R. A. DelGiudice
X5045	Swine, nasal	R. F. Ross	R. F. Ross
75151	Guinea pig, genital tract	E. V. Langford	E. V. Langford
Acholeplasma axanthum			
S-743	Tissue culture	C. Friend	J. G. Tully
1190	Lagoon	R. F. Ross	R. F. Ross
D-1	Swine, joint	D. Schimmel	L. Stipkovits
H86N	Bovine, nasal	N. O. Olson	J. G. Tully
1025C	Kale	N. L. Somerson	N. L. Somerson
0501	Broccoli	N. L. Somerson	N. L. Somerson
J544	Coconut palm	S. J. Eden-Green	S. J. Eden-Green
J248	Coconut palm	S. J. Eden-Green	S. J. Eden-Green
Acholeplasma laidlawii	Sewage	P. P. Laidlaw & W. J. Elford	D. G. ff. Edward
Mycoplasma gallisepticum			
PG31 (x95)	Chicken, trachea	F. S. Markham	Reference reagent NIH
S6	Turkey, brain	D. V. Zander	J. Noel
F(R)	Chicken, trachea	R. Yamamoto & H. E. Adler	J. Fabricant
293-1	Chicken, trachea	J. Taylor	J. Fabricant
Mycoplasma genitalium			
G-37*	Man, genital tract	J. G. Tully & D. Taylor-Robinson	J. G. Tully
G-37 (chimp)†	Chimpanzee, genital tract (experimentally infected)	J. G. Tully & M. F. Barile	J. G. Tully
M-30‡	Man, genital tract	J. G. Tully & D. Taylor-Robinson	J. G. Tully

* Isolated from the urethra of a non-gonococcal urethritis patient in SP4 broth at 37 °C (Tully *et al.*, 1981). Two cloned cultures were tested, one at passage level 8 and the other at 17.

† Isolated on SP4 medium (passage 1) from the urethra of a chimpanzee 8 weeks after experimental intra-urethral infection with the G-37 cloned strain at passage 8.

‡ Isolated from the urethra of another non-gonococcal urethritis patient on SP4 broth at 30 °C, cloned and tested at the fourth passage.

*Hin*dIII, and 50 mM-Tris/HCl, pH 7·5, 10 mM-MgSO₄ and 100 mM-NaCl for *Xho*I. For the enzymes *Hpa*I and *Kpn*I, the salt solution was composed of 10 mM-Tris/HCl, pH 7·5, and 10 mM MgSO₄, and for *Sma*I, the solution contained in addition 20 mM-KCl. A reaction mixture containing the tested endonuclease, the appropriate salt solution and 1 µg of phage lambda DNA (Bethesda Research Laboratories, BRL, Gaithersburg, Md.) served as a control of specific enzyme activity. Digestions were carried out at 37 °C for 60 min. The reaction was stopped by adding 2 µl of tracking dye solution consisting of 0·07% (w/v) bromophenol blue, 7% (w/v) SDS, and 33% (v/v) glycerol in water.

Electrophoresis of digestion products. The total volume of the reaction mixture was subjected to electrophoresis in slab gels made of 1% (w/v) agarose (Type 1, Low EEO; Sigma) prepared in Tris/borate buffer (89 mM-Tris, pH 8·2, 2·5 mM-EDTA, and 89 mM-boric acid) used also as electrophoresis buffer. A vertical electrophoresis apparatus (BRL, model V16) was used at 100 volts for 3 h until the dye marker reached the bottom of the gel. The gel was stained for 15 min with ethidium bromide (0·4 µg ml⁻¹) and photographed under UV light (Chromato-Vue transilluminator model C-61, Ultra-Violet Products, San Gabriel, Calif.) using Polaroid type 55 P/N film and a Polaroid MP-4 land camera with an orange filter. The negatives obtained were used to prepare prints in which the polynucleotide bands appear dark on a light background. A control preparation of undigested DNA (DNA preparations without the addition of restriction endonucleases) from each mycoplasma was also subjected to electrophoresis to detect extrachromosomal DNA. Only one chromosomal band was seen with no evidence of extrachromosomal DNA by the electrophoretic procedure.

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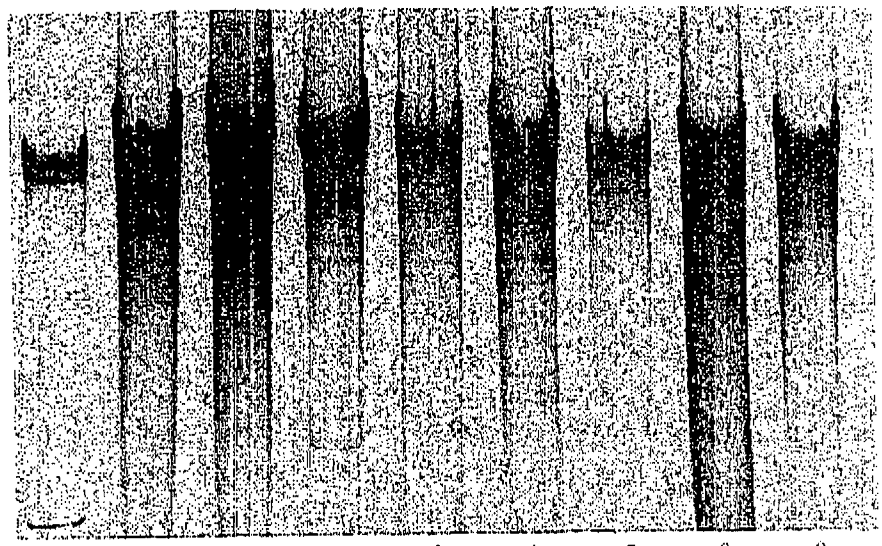
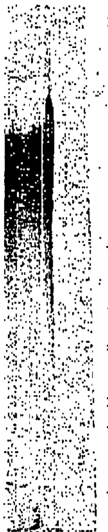


Fig. 1. Cleavage patterns of the DNA of *A. axanthum* strains (lanes 2–9) and of phage lambda DNA (lane 1) digested by *Kpn*I. The strains tested were S-743 (lane 2); 0501 (lane 3); D-1 (lane 4); 1025C (lane 5); H86N (lane 6); J544 (lane 7); 1190 (lane 8) and J248 (lane 9). Of the eight strains tested, the patterns of only strains 0501 and 1025C (both isolated from vegetables) appear similar. The 'smears' seen in the gels indicate that some degradation of DNA occurred during DNA extraction.

RESULTS

The DNA cleavage patterns of *Acholeplasma axanthum* strains, particularly those obtained with restriction endonucleases with recognition sites rich in G + C, support the intraspecies genetic heterogeneity of this species indicated by the DNA hybridization data (Stephens *et al.*, 1983). Thus, of the eight *A. axanthum* strains tested only the DNA of strains 0501 and 1025C (both isolated from vegetables, Table 1) produced identical cleavage patterns with *Kpn*I (recognition site GGTAC/C) and with *Bam*HI (recognition site G/GATCC), whereas the DNAs of the other six strains each yielded a different pattern (Figs 1 and 2). On the other hand, the cleavage patterns of the DNAs of the five *A. oculi* strains by *Kpn*I and *Bam*HI, though not being identical, resembled each other (Fig. 3). In addition, Fig. 3 shows that the *A. oculi* DNAs can be distinguished from those of *A. axanthum* in being cleaved at many more points when digested with *Kpn*I, and the cleavage pattern of the *A. laidlawii* PG8 DNA by this enzyme differs from the patterns of the *A. axanthum* and *A. oculi* strains. *Pst*I, despite sharing with *Bam*HI and *Kpn*I a recognition site rich in G + C (CTGCA/G) cleaved the acholeplasmal DNAs at many points, producing highly similar patterns with the *A. oculi* strains, but different patterns with the *A. axanthum* and *A. laidlawii* strains (Fig. 4).

The restriction endonucleases *Hpa*I and *Hin*dIII which have recognition sites poor in G + C (GTT/AAC and A/AGCTT, respectively) cut the G + C poor acholeplasmal DNA at many sites, producing cleavage patterns with numerous bands, making comparison of patterns more difficult. Again, the patterns of the *A. axanthum* strains obtained with these enzymes differed from each other, as against the similarity of patterns exhibited by the selected *A. oculi* strains tested (data not shown). On the other hand, digestion of the acholeplasmal DNAs by *Xho*I (recognition site C/TCGAG) produced patterns with no or very few visible cleavage bands, rendering this enzyme not useful for our purpose. *Sma*I, the enzyme with a recognition site CCC/GGG, did not produce any visible cleavage fragments when applied to the DNAs of the five *A. oculi* strains, and three of the *A. axanthum* strains (D-1, 1190, and S743) but produced four visible cleavage bands with *A. laidlawii* PG8 DNA (data not shown).



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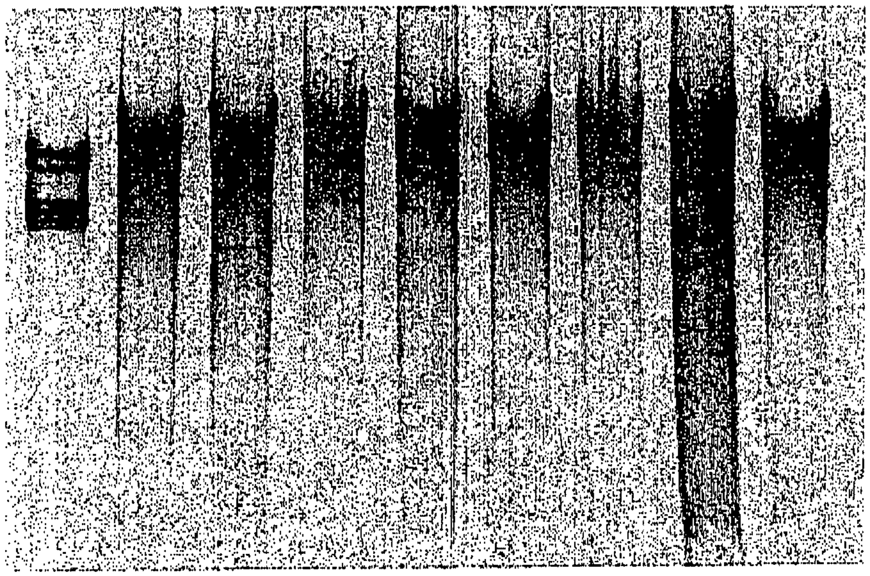

Fig. 2. Cleavage patterns of the DNA of *A. axanthum* strains (lanes 2–9) and of phage lambda DNA (lane 1) digested by *Bam*HI. The order of the strains in the lanes is as depicted in Fig. 1. Only strains 0501 and 1025C produced similar or identical patterns.

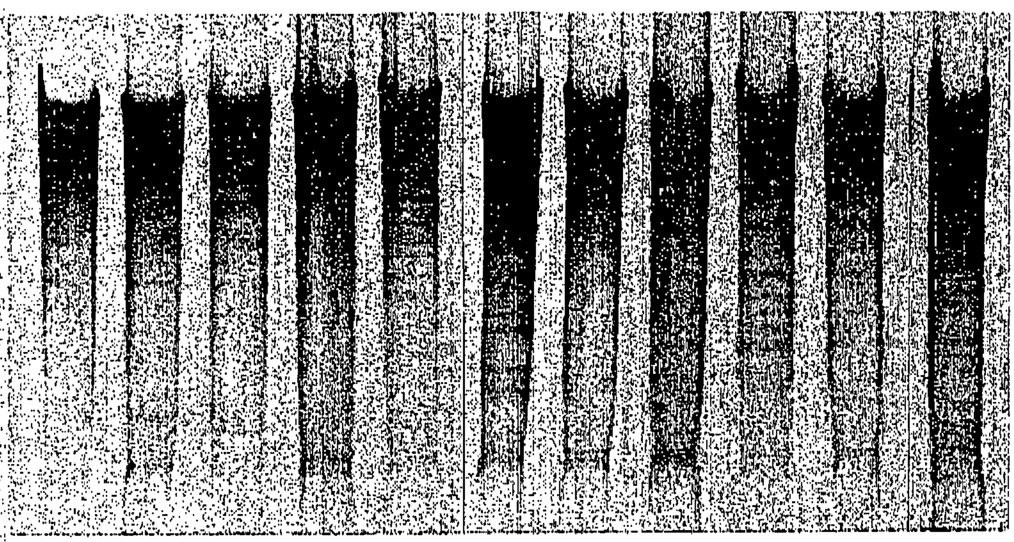

Fig. 3. Cleavage patterns of the DNA of *A. oculi* strains by *Bam*HI (lanes 1–5) and by *Kpn*I (lanes 6–10). The cleavage pattern of *A. laidlawii* PG8 DNA by *Kpn*I is included for comparison in lane 11. The *A. oculi* strains tested were: 19L (lane 1); 3557TC (lanes 2 and 6); goat 5 (lanes 3 and 7); 70-538TC (lanes 4 and 8); X5054 (lanes 5 and 9) and 75151 (lane 10).

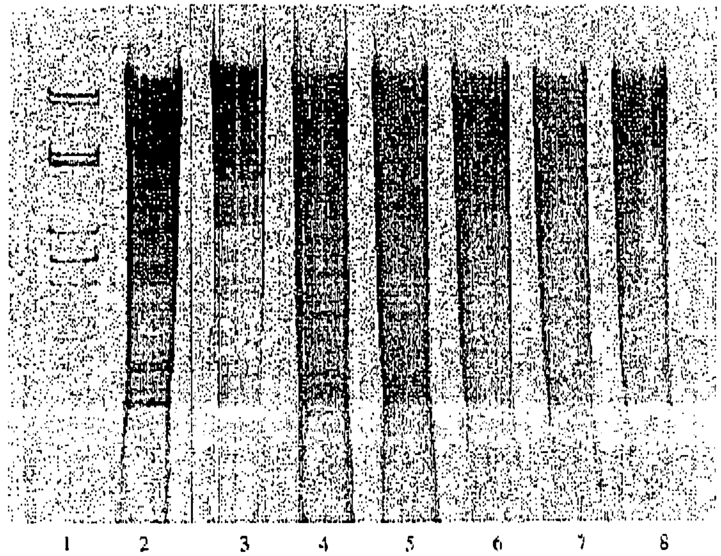

Fig. 4. Cleavage patterns of phage lambda DNA (lane 1) and the DNAs of *A. laidlawii* PG8 (lane 2), *A. axanthum* D-1 (lane 3) and *A. oculi* strains (lanes 4–8) digested by *Pst*I. The *A. oculi* strains tested were: X5045 (lane 4); 70-538TC (lane 5); goat 5 (lane 6); 3557TC (lane 7) and 19L (lane 8). The marked similarity of the *A. oculi* patterns to each other, and their dissimilarity to the *A. axanthum* and *A. laidlawii* patterns can be seen.

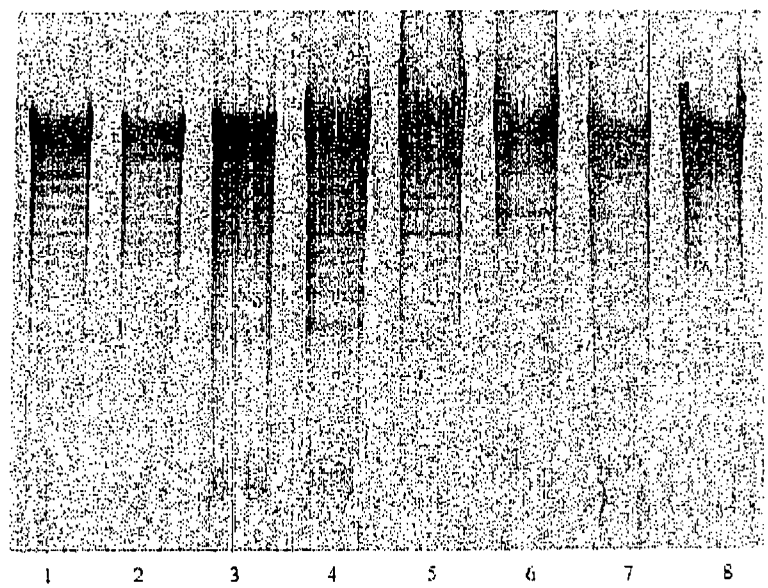

Fig. 5. Cleavage patterns of the DNA of *M. gallisepticum* strains by *Bam*HI (lanes 1–4) and by *Kpn*I (lanes 5–8). The strains tested were: PG-31 (lanes 1 and 5); S6 (lanes 2 and 6); F(R) (lanes 3 and 7) and strain 293-1 (lanes 4 and 8). The marked similarity of the patterns is particularly noticeable with *Bam*HI. The patterns of strains PG-31 and 293-1 are practically identical with both enzymes.

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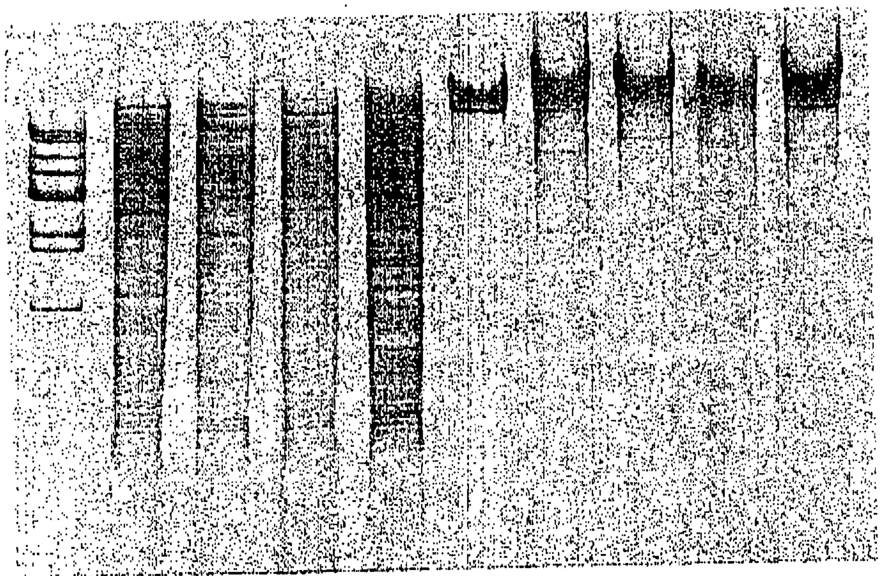

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Fig. 6. Cleavage patterns of lambda phage DNA and DNAs of *M. gallisepticum* strains by *Hpa*I (lanes 1–5) and by *Xho*I (lanes 5–10). The strains tested: PG-31 (lanes 2 and 7); S6 (lanes 3 and 8), F(R) (lanes 4 and 9) and 293-1 (lanes 5 and 10). The essential identity of the patterns of strains PG-31 and 293-1 can be seen.

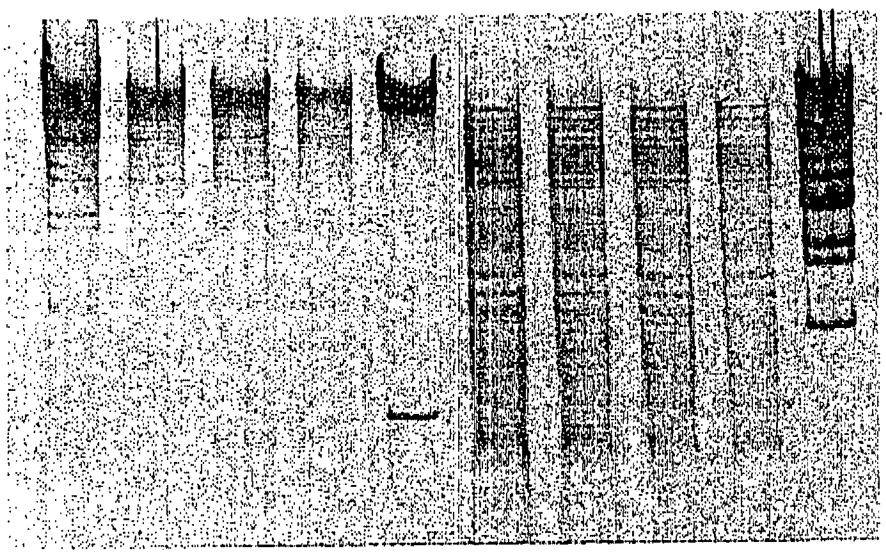

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Fig. 7. Cleavage patterns of the DNA of *M. genitalium* strains and of phage lambda DNA by *Kpn*I (lanes 1–5) and by *Hpa*I (lanes 6–10). The strains tested: G-37, passage 17 (lanes 1 and 6); G-37, passage 8 (lanes 2 and 7); G-37 isolated from the urethra of an experimentally infected chimpanzee (lanes 3 and 8); M-30, passage 4; lambda phage DNA (lanes 5 and 10). The identity of the cleavage patterns of all strains can be seen with both enzymes.

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Based on the known close serologic relatedness, similarities in electrophoretic patterns of cell proteins (Razin & Rottem, 1967; Rhoades *et al.*, 1974) and narrow host specificity of *M. gallisepticum* strains (Jordan, 1979) we had predicted that the cleavage patterns of the DNAs of the various *M. gallisepticum* strains would be similar. This, indeed, proved to be the case, as the cleavage patterns of the DNAs of the four *M. gallisepticum* strains by *Bam*HI (Fig. 5), *Hpa*I (Fig. 6) and *Pst*I (data not shown) were almost identical. Nevertheless, the cleavage patterns obtained with *Xho*I and *Kpn*I, enzymes which cut the *M. gallisepticum* DNA at fewer points, revealed some differences among the strains, although PG-31 and 293-1 appeared identical in all cases (Figs 5 and 6). As with the *A. axanthum* and *A. oculi* DNAs, *Sma*I failed to produce visible cleavage bands with the *M. gallisepticum* DNA, apart from one band with the S6 DNA (data not shown). Genetic homogeneity was even more striking with the human genital *M. genitalium* strains. The cleavage patterns by *Kpn*I and *Hpa*I of the DNA of strains G-37 and M-30 isolated from the urethra of different patients (Tully *et al.*, 1981) were essentially identical (Fig. 7). Moreover, the organisms recovered from a chimpanzee 8 weeks after it had been inoculated with *M. genitalium* strain G-37, exhibited DNA cleavage patterns identical with those of the original strain (Fig. 7). The same results were obtained with *Bam*HI and *Pst*I (results not shown).

DISCUSSION

The working hypothesis that genetic heterogeneity among strains of the same species is associated with their recovery from diverse habitats has been put forward by Stephens *et al.* (1983) on the basis of DNA hybridization data. Accordingly, the hypothesis would suggest that adaptation of an organism to a new host had pressured it to genotypically change in order to survive or that colonization occurred through a selective process. The DNA cleavage data reported here support the above notion by demonstrating the marked genotypic heterogeneity of the *A. axanthum* strains isolated from a variety of hosts (Table 1). The *A. oculi* strains, though appearing closer in DNA structure to each other than the *A. axanthum* strains, still exhibit significant variations in their cleavage patterns, a fact which may be related to the variety of hosts from which the *A. oculi* strains were isolated (Table 1). Unfortunately, in the case of *A. oculi* DNA hybridization data are unavailable for comparison with the DNA cleavage data.

In contrast to the marked heterogeneity of the two *Acholeplasma* species, the DNA cleavage patterns of *M. gallisepticum* strains indicated marked genetic homogeneity, despite the fact that the strains were isolated in different laboratories, and were subcultured numerous times. It can be argued in this case that *M. gallisepticum* has a much more restricted ecological niche than the acholeplasmas, as it only inhabits the respiratory tract of chickens and turkeys (Jordan, 1979). In genetic homogeneity, *M. gallisepticum* resembles another avian pathogen *M. meleagridis* (Elmahi *et al.*, 1982) and the human respiratory pathogen *M. pneumoniae* (Chandler *et al.*, 1982; Razin *et al.*, 1983). In the case of *M. pneumoniae* the ecological niche is even more restricted, as this mycoplasma is found in the respiratory tract of man only. *Mycoplasma pneumoniae* has not been isolated under natural conditions from any other animal, including primates (Barile, 1973). Data on the occurrence of *M. genitalium* are still very scarce. Nevertheless, the DNA cleavage patterns suggest that this mycoplasma, considered as a potential pathogen in the urogenital tract (Tully *et al.*, 1981; Taylor-Robinson *et al.*, 1981) constitutes another genotypically homogeneous species.

DNA cleavage data are available for another human mycoplasma, *Ureaplasma urealyticum* (Razin *et al.*, 1983). In this case, the cleavage patterns, as well as DNA hybridization data and electrophoretic patterns of cell proteins, indicate the presence of two genetically distinct clusters of strains. However, as against the marked diversity exhibited by the *A. axanthum* strains, the strains in each of the two *U. urealyticum* clusters show remarkable homogeneity.

The experience gained so far from the use of restriction endonucleases for classification purposes enables the drawing of some conclusions as to the advantages and deficiencies of this approach. The advantages include: (1) Need for a small quantity of organisms, as only a few μg of DNA suffice for each test. (2) No interference by culture medium components particularly proteins, a problem complicating the electrophoretic analysis of cell proteins of poorly-growing

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mycoplasmas in serum-rich media (see Razin *et al.*, 1983). (3) Easy reading of results based on visual comparison of patterns. Densitometer tracings of the patterns can also be prepared (Bove *et al.*, 1982). (4) The DNA cleavage patterns are excellent tools to test for genetic homogeneity. Identical patterns obtained by several restriction enzymes strongly indicate genetic homogeneity of subcultures. The case of the *M. genitalium* strains illustrates this point. Also, the susceptibility of the DNA of a tested strain to cleavage by restriction endonucleases with G + C rich recognition sites, provides an indication for assessment of the G + C content of this particular DNA. Thus, the relatively G + C rich *M. pneumoniae* DNA (G + C about 40 mol%) is cleaved by *Sma*I (recognition site CCC/GGG) at about 35 sites, whereas the DNAs of other *Mycoplasma* and *Acholeplasma* species, which are poorer in G + C, usually resist digestion by this enzyme (present results and Razin *et al.*, 1983).

Possible deficiencies of the DNA cleavage approach can be summarized as follows: (1) Undegraded DNA of high purity is required. This is not easy to obtain with the *Acholeplasma* species, as they are particularly rich in endogenous DNAases which are activated on cell lysis (Razin *et al.*, 1964; Pollack *et al.*, 1965). We found that by increasing the EDTA concentration in the lysis solution, and by processing relatively small pellets of cells, the self-digestion of the mycoplasmal DNA can be minimized. We have not encountered this problem with *Mycoplasma* species. (2) The presence of extrachromosomal DNA, such as that of plasmids or viruses, complicates the interpretation of cleavage patterns (Bove *et al.*, 1982). It can be argued that the cleavage patterns of *A. axanthum* strains are so different from each other due to the presence of different plasmids and/or viruses in these strains. Although this possibility cannot be totally ruled out, electrophoresis of undigested DNA of these strains failed to show any bands additional to that of the chromosomal DNA. (3) Comparison of cleavage patterns is done visually by direct comparison of bands on photographs or on densitometer tracings of the photographs. This suffices for determining complete identity, close-similarity, or total non-identity of DNA structure. The problem lies in cases of partial similarity, as the cleavage data are not expressed by numbers, such as percent homology provided by DNA hybridization tests.

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